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Differential Expression of Chitinases in *Vitis vinifera* L. Responding to Systemic Acquired Resistance Activators or Fungal Challenge[1]

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The concept of systemic acquired resistance (SAR) enables a novel approach to crop protection, and particular pathogenesis-related proteins, i.e. an acidic chitinase, have been classified as markers of the SAR response. Basic class I (VCHIT1b) and a class III (VCH3) chitinase cDNAs were cloned from cultured *Vitis vinifera* L. cv Pinot Noir cells and used to probe the induction response of grapevine cells to salicylic acid or yeast elicitor. Furthermore, the cells were treated with the commercial SAR activators 2,6-dichloroiso-nicotinic acid or benzo(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester. Elicitor or salicylic acid induced both VCHIT1b and VCH3 transcript abundances, whereas 2,6-dichloroiso-nicotinic acid or benzo(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester enhanced exclusively the expression of VCH3. To assess the systemic sensation of chitinase expression, single leaves of *Vitis vinifera* L. cv Pinot Noir or *Vitis rupestris* plants were inoculated with *Plasmopara viticola* spore suspensions, and the VCH3 and VCHIT1b mRNA amounts in the infected versus the adjacent, healthy leaf were monitored. Two VCH3 mRNA maxima were observed 2 and 6 d postinoculation in the infected, susceptible *V. vinifera* tissue, whereas in the healthy leaf the transcript increased from low levels d 2 postinoculation to prominent levels d 6 to 8 postinoculation. The level of VCH3 mRNA increased also over 4 d in the inoculated, resistant *V. rupestris* tissue. However, necrotic spots rapidly limited the infection, and the VCH3 transcript was undetectable in the upper-stage, healthy leaf. The expression of VCHIT1b remained negligible under either experimental condition. Overall, the results suggest that the selective expression of VCH3 might be a reliable indicator of the SAR response in *V. vinifera* L.

Field-grown crops are permanently endangered by fungal pathogens, and extensive farming requires the persistent control of pathogenic fungal populations. Synthetic fungicides are commonly used for pest control, which affect the inoculum directly and have to be applied frequently throughout the season, depending on the fungal pressure. Moreover, the action spectrum of fungicides is often selective and requires the combination of several active compounds under the auspices of an integrated pest management. A promising alternative that has emerged recently is based primarily on strengthening the endogenous defense capabilities of plants (Kessmann et al., 1997). Plants are known to develop an immune-like response (SAR) to local fungal challenge, which disseminates through the tissues in advance of the fungus (Kuc, 1982; Malamy et al., 1990; Kessmann et al., 1994; Ryals et al., 1996). The SAR response is probably based on multiple mechanisms that can lead to long-term and broad-spectrum disease control, and SA was proposed to play a pivotal role in this process by mediating the local rather than the systemic transmission of signals (Metraux et al., 1991; Malamy and Klessig 1992; Gaffney et al., 1993; Kessmann et al., 1997). Unfortunately, the response to local fungal infections could hardly be exploited for crop protection, since it is unpredictable in timing and level of expression (Kessmann et al., 1997). However, recently developed synthetic chemicals such as INA and BTH have a priming effect and induce the SAR response in various test plants (Metraux et al., 1991; Friedrich et al., 1996; Kessmann et al., 1997). These "plant activators" appear to replace SA in the pathway leading to SAR (Lawton et al., 1996), and their effect is dose dependent.

Fungal challenge of plants causes multiple metabolic changes, and the accumulation of PRPs in the intercellular space was initially considered unique (Van Loon, 1985). Later, PRPs were also recovered from the intracellular compartment (Linthorst, 1991; Kombrink and Somssich, 1995), and the ubiquitous distribution of these proteins in plants as well as their highly conserved sequences were soon realized. In view of the lack of a particular physiological function, the common features of classification recognized early were the protease resistance and stress inducibility (Ohashi and Oshima, 1992). However, the effective induction of PRPs was also accomplished by low concentrations of SA (Pierpoint et al., 1990), suggesting the conservation of SA-responsive, *cis*-active promoter elements (Goldsbrough et al., 1993).

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Abbreviations: BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester; INA, 2,6-dichloroisonicotinic acid; PRP, pathogenesis-related protein; SA, salicylic acid; SAR, systemic acquired resistance.

Meanwhile, some PRPs have been identified as chitinases or β-1,3-glucanases (Kauffmann et al., 1987; Legrand et al., 1987; Kombrink et al., 1988; Joosten and de Wit, 1989), and specific promoter motifs were identified (Fukuda and Shinshi, 1994). Chitinase genes are known to be regulated in a developmental and organ-specific pattern, but stress conditions such as fungal challenge, elicitor treatment, or exposure to ethylene also induced their expression (Graham and Sticklen, 1994; Kombrink and Somssich, 1995). In the case of fungal inoculation, the enhanced chitinase activity was functionally implicated in the defense response directed toward chitin as a major cell wall component of most fungi (Schlumbaum et al., 1986; Mauch et al., 1988). The combination of chitinase with β-1,3-glucanases was proposed to further potentiate the antifungal activity and was shown experimentally to inhibit the growth of many pathogenic fungi (Sela-Buurlage et al., 1993). Some chitinases inherited lysozyme activity and these enzymes may be required for the defense against bacterial pathogens (Metraux et al., 1989; Majeau et al., 1990).

Five classes of plant chitinases were distinguished on the basis of sequence alignments and subcellular topology (Melchers et al., 1994), and three of these are ubiquitously found in plants (Shinshi et al., 1990; Meins et al., 1992; Graham and Sticklen, 1994; Kombrink and Somssich, 1995). Class I chitinases are of basic pI and contain an N-terminal Cys-rich domain, which supposedly functions in chitin binding. Furthermore, a hinge domain was assigned, and a C-terminal extension was identified that is essential and sufficient for vacuolar localization (Neuhaus et al., 1991). Class II chitinases, which share highly conserved catalytic domains with class I, are distinguished by their acidic pI and lack of Cys-rich domains or C-terminal extension and are excreted into the extracellular space. Class III chitinases are characterized by lysozyme activity and insignificant sequence homologies with either class I or class II chitinases. At least in *Parthenocissus* sp. and cucumber (*Cucumis sativus*), the class III chitinases appear to be localized in the extracellular matrix (Bernasconi et al., 1987; Boller and Metraux, 1988). The antifungal potential of class III chitinases is unclear (Melchers et al., 1994).

Tobacco (*Nicotiana tabacum*) or Arabidopsis plants express the SAR upon fungal inoculation (Ward et al., 1991; Uknes et al., 1992), and the systemic response was tightly correlated in tobacco with the induced expression of at least nine gene families, including class III chitinases (Ward et al., 1991; Ryals et al., 1996). Local and systemic de novo expression of class III chitinase was also induced by tobacco mosaic virus infection of tobacco (Lawton et al., 1992). Furthermore, treatment of tobacco and cucumber with SA or INA also caused the strong expression of class III chitinase (Ward et al., 1991; Lawton et al., 1994) and the results obviously suggest that class III chitinase transcripts can be considered as molecular markers of the SAR response. Nevertheless, specific correlations of chitinase expression with SAR were recorded for only a limited number of test plants and need to be verified for most crop plants (Ryals et al., 1996). *Vitis vinifera* L., cultivated worldwide in warm climates since ancient times, is particularly sensitive in Europe to *Plasmopara viticola* and *Uncinula necator*, the causal agents of downy and powdery mildew diseases. These diseases are difficult to control and have caused severe losses on several occasions. Despite the economic relevance, surprisingly little basic information is available concerning PRPs or the SAR capabilities of grapevine. We therefore initiated model studies in *V. vinifera* L. cv Pinot Noir cell-suspension cultures to identify the primary structure and expression of class I and class III chitinases in response to fungal elicitation or treatment with commercial plant SAR activators. These studies were extended to *V. vinifera* L. and *Vitis rupestris* plants inoculated with *P. viticola* to prove the systemic differential induction of the chitinases.

MATERIALS AND METHODS

Chemicals, Enzymes, and Materials

Restriction enzymes, vectors, *Escherichia coli* host strains, and biochemicals were purchased from Boehringer Mannheim (Pensberg, Germany), MBI Fermentas (St. Leon-Rot, France), and Stratagene. [α-^{32}P]dATP was bought from Amersham. All other chemicals were purchased from Sigma or Roth (Karlsruhe, Germany). INA and BTH as 25% (or 50%) active ingredients in "wettable powder" and wettable powder lacking the chemicals were kindly supplied by H. Kessmann and M. Oostendorp (Novartis, Basel, Switzerland). Tobacco (*Nicotiana tabacum*) class I and class III chitinase cDNA clones (Shinshi et al., 1990; Stintzi et al., 1993) as well as the corresponding antisera were kindly provided by M. Legrand (Centre National de la Recherche Scientifique, Strasbourg, France). The synthesis of oligonucleotides was carried out by G. Igloi (Institut für Biologie III, Freiburg).

Plant Materials and Cell Cultures

Plants of *Vitis vinifera* L. cv Pinot Noir and *Vitis rupestris* were grown to a height of 0.6 to 0.8 m in the greenhouse at 28/23°C day and night cycles. Single leaves at the middle leaf stage of the intact plants were inoculated with *Plasmopara viticola*. Tissue- and cell-suspension cultures of *V. vinifera* L. cv Pinot Noir were propagated as described elsewhere (Busam et al., 1997).

Fungal Inoculation

Grapevine plants were infected with freshly collected *P. viticola* (Berk. & Curt. ex de Bary) Berl. & de Toni spores that were resuspended in water (approximately 2.5×10^4 spores mL^{-1}) and sprayed as a fine mist to the point of imminent runoff on the underside of leaves. The leaves were incubated overnight in wet polyethylene bags at approximately 100% RH and 23°C. The bags were removed subsequently and the locally infected leaves as well as the younger, next stage healthy leaves were collected at time intervals. Harvested tissues were frozen in liquid nitrogen and stored at −70°C.

Induction of Cell Cultures

Ten-day-old cultures were treated with either yeast extract (1 mg mL^{-1}; Difco, Detroit, MI), live *Pseudomonas syringae* pv *syringae* (D20) cells (1×10^9 cells/40 mL culture), sterile-filtered aqueous solutions of SA (final concentration 20 μM), or suspensions of INA and BTH, formulated as 25 and 50% active ingredients in wettable powder, respectively. The final concentrations of INA or BTH in the grapevine cell-suspension cultures were 25 μM. Wettable powder lacking INA or BTH at a final concentration of 0.15 mg/40 mL culture served as a control. The cells were harvested by vacuum filtration at time intervals of the induction, frozen immediately in liquid nitrogen, and stored at −70°C until use.

cDNA Cloning

A cDNA library in UNI-ZAP (Stratagene) was established from poly(A^+) RNA (5 μg) of cultured *V. vinifera* L. cv Pinot Noir cells that had been elicited for 4 h with yeast extract, and one clone was selected by three rounds of plaque-hybridization screening at low stringency of approximately 2×10^5 recombinants using a ^{32}P-labeled tobacco class I chitinase cDNA probe (Shinshi et al., 1990). The λZAP clone VCHIT1b rescued by in vivo excision harbored an insert of approximately 1.2 kb. Hybridization screening of the cDNA library with a tobacco class III chitinase cDNA (Stintzi et al., 1993) failed, and a *Vitis*-specific class III chitinase cDNA probe was generated by reverse-transcription-PCR amplification. Poly(A^+) RNA (1 μg) from cultured *V. vinifera* cells that had been elicited for 4 h with yeast extract was used as a template for amplification with degenerate oligonucleotide primers complementary to conserved peptide regions of class III chitinases. The combination of the sense primer 5′-TAT TGG GGC/T CAA AAC/T GGC/A/G AAC/T GA-3′, corresponding to the peptide motif YWGQNGNE, and the antisense primer 5′-GG A/GTT A/GTT GTA A/GAA TTG AAT/C CCA-3′, derived from the peptide W(I/V)QFYNNP, amplified a 550-bp fragment, which was subcloned in pBluescript KS II and sequenced for identification. Five clones were selected from the *Vitis* sp. cDNA library in three rounds of hybridization screening using the ^{32}P-labeled cDNA probe under stringent conditions. The plasmids (pBluescript SK^+) of the VCH3 clones were rescued following the in vivo excision protocol for λZAP (Stratagene) and were shown to harbor inserts of approximately 1.1 kb. Both strands of the double-stranded DNA were sequenced by the dideoxy-chain-termination method (Sanger et al., 1977) using modified T7 DNA polymerase (Sequenase, United States Biochemical) and combinations of universal (M13) or reverse sequencing (RP) primer in addition to sequence-derived primers.

Northern-Blot Hybridization

Total RNA was isolated from deep-frozen *Vitis* sp. cells according to the method of Chang et al. (1993). Poly(A^+) RNA of the cells was isolated using oligo(dT)-magnetic microspheres (Dynal, Hamburg, Germany) according to the manufacturer's recommendations or to the work of Hankanes et al. (1993). The RNA was stored at −20°C in water until use and quantified spectrophotometrically (Gene-quant, Pharmacia). Equivalent amounts of RNA were denatured in formamide/formaldehyde and separated by electrophoresis on 1.2% agarose gels in the presence of formaldehyde and ethidium bromide (0.5 μg mL^{-1}) prior to blotting on nylon membranes (Sambrook et al., 1989). Equal loading of samples was verified after the gel was run under UV irradiation and photographic documentation. The chitinase cDNAs VCHIT1b and VCH3 were labeled with digoxigenin-11-dUTP using the DIG-High Prime labeling kit (Boehringer Mannheim) according to the manufacturer's recommendations. Hybridization with either one of these probes was carried out at 42°C overnight in a solution containing 50% formamide. The hybridization signals were spotted after stringent washings with disodium 4-chloro-3-(methoxyspiro{1,2-dioxetane-3, 2′-(5′-chloro)-tri-cyclo[3.3.1.1]decan}-4-yl)-phenyl phosphate (Boehringer Mannheim) as the substrate following the manufacturer's recommendations. The membranes were stripped for 20 min in boiling buffer containing 0.01× SSPE and 0.1% SDS and subsequently washed twice with a solution of 2× SSC and 0.1% SDS at room temperature before rehybridization.

Southern Hybridization

Genomic DNA was isolated from the leaves of 8-week-old *V. vinifera* L. cv Pinot Noir plantlets (Steenkamp et al., 1994) and samples of 10 μg were digested with one of the endonucleases *Eco*RI, *Pst*I, or *Hin*dIII or with both *Eco*RI and *Pst*I. The restriction fragments were separated by electrophoresis on a 0.7% agarose gel and blotted by downward capillary transfer (Zhou et al., 1994) to nylon membranes. The blots were hybridized with digoxigenin-labeled VCHIT1b or VCH3 cDNAs at 65°C overnight, washed subsequently under stringent conditions (Sambrook et al., 1989), and developed with disodium 4-chloro3-(methoxyspiro{1,2-dioxetane-3, 2′-(5′-chloro)-tricyclo[3.3.1.1]decan}-4-yl)phenyl phosphate (Boehringer Mannheim) according to the manufacturer's recommendations.

RESULTS

Chitinase cDNAs

Cell-suspension cultures of *V. vinifera* L. cv Pinot Noir were established for model investigations of the mechanisms involved in the inducible grapevine disease resistance response (Busam et al., 1997). Cells elicited for 4 h with yeast extract served as a source of poly(A^+) RNA for the construction of a cDNA library in UNI-ZAP, and one clone, designated VCHIT1b, was isolated from this library by hybridization screening with a class I chitinase cDNA from tobacco (Shinshi et al., 1990). This clone harbored an insert of 1124 bp, which was fully sequenced and shown to contain only one long open reading frame, spanning 942 bp, flanked by stretches of 11 and 171 bp at the 5′ and 3′

ends, respectively (Fig. 1). The open reading frame encoded a polypeptide of 314 amino acid residues, which revealed a molecular mass of 33,428 D for the translated polypeptide (Fig. 1). Three stop codons and two putative polyadenylation sites were recognized in the 3′ flanking region, which were followed by a short poly(A) tail (Fig. 1).

Sequence alignments of VCHIT1b with heterologous class I chitinases (Gaynor, 1988; Parsons et al., 1989; Shinshi et al., 1990) indicated high degrees of identity of about 70 and 53 to 70% at the DNA and peptide level, respectively (Fig. 2), clearly suggesting that the cDNA cloned from *V. vinifera* encoded a class I chitinase. Moreover, sequence domains typical for this class of chitinase, such as a hydrophobic signal peptide of 20 amino acid residues followed by a Cys-rich region (8 conserved Cys residues) with homology to wheat germ agglutinin (Raikhel and Wilkins, 1987), were recognized in the polypeptide sequence (Fig. 2). The signal peptide is likely to be required for intracellular trafficking of the enzyme to the ER, and the Cys-rich domain was proposed to be essential for high chitinase activity (Graham and Sticklen, 1994). Nevertheless, the hinge domain of only three amino acid residues, SST, preceding the catalytic domain in VCHIT1b was comparatively short and lacked the more common Pro or Gly residues, whereas the major catalytic domain was strictly conserved in comparison with heterologous class I chitinases (Meins et al., 1992; Fig. 2). In addition, VCHIT1b encoded a conserved C-terminal element of at least seven amino acid residues, which is sufficient for trafficking of the enzyme to the vacuole (Neuhaus et al., 1991). Thus, VCHIT1b is conceivably deposited in grapevine vacuoles, and the presence of the C-terminal extension and the Cys-rich domain as well as the pI of 7.90 classify the enzyme as a basic type class I chitinase.

Analogous hybridization screenings of the *V. vinifera* cDNA library with a class III chitinase cDNA probe from tobacco (Stintzi et al., 1993) failed to pick out corresponding clones. Therefore, the reverse-transcription-PCR amplification of a grapevine-specific cDNA probe was pursued using two cDNA primers complementary to highly conserved peptide sequences of heterologous class III chitinases and a poly(A^+) RNA template from elicited *V. vinifera* cells. The PCR-amplified 550-bp DNA fragment was sequenced and revealed approximately 66% identity to class III chitinases from tobacco, cucumber, and Arabidopsis (Metraux et al., 1989; Samac et al., 1990; Stintzi et al., 1993). Stringent hybridization screenings of the *V. vinifera*

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1                                                                  GGCACGAGGAA

12      ATG GGG TTG TGG GCA TTG GTA GCT TTC TGT CTG TTG TCA TTA ATA
        M  G  L  W  A  L  V  A  F  C  L  L  S  L  I                  15

57      CTG GTT GGC TCA GCA GAG CAA TGT GGA GGG CAA GCT GGG GGT AGA
        L  V  G  S  A  E  Q  C  G  G  Q  A  G  G  R                  30

102     GTT TGC CCA GGG GGG GCA TGC TGC AGC AAG TTT GGT TGG TGT GGC
        V  C  P  G  G  A  C  C  S  K  F  G  W  C  G                  45

147     AAC ACT GCT GAT TAC TGT GGC AGT GGC TGC CAA AGC CAG TGC AGT
        N  T  A  D  Y  C  G  S  G  C  Q  S  Q  C  S                  50

192     TCC ACT GGT GAC ATT GGC CAG CTT ATT ACC AGG TCC ATG TTC AAT
        S  T  G  D  I  G  Q  L  I  T  R  S  M  F  N                  65

237     GAT ATG CTT AAG CAT AGA AAT GAG GGG AGT TGC CCT GGC AAG GGC
        D  M  L  K  H  R  N  E  G  S  C  P  G  K  G                  80

282     TTC TAC ACC TAT GAC GCT TTC ATA GCT GCT GCT AAG GCC TTT CCT
        F  Y  T  Y  D  A  F  I  A  A  A  K  A  F  P                  95

327     GGC TTT GGA ACA ACT GGT GAT ACC ACT ACT CGT AAA AGG GAA ATC
        G  F  G  T  T  G  D  T  T  T  R  K  R  E  I                 110

372     GCA GCC TTC TTG GCT CAA ACT TCT CAT GAA ACC ACT GGG GGG TGG
        A  A  F  L  A  Q  T  S  H  E  T  T  G  G  W                 125

417     GCT AGT GCA CCT GAT GGC CCA TAC GCT TGG GGA TAC TGC TAC CTC
        A  S  A  P  D  G  P  Y  A  W  G  Y  C  Y  L                 140

462     AGG GAA CAA GGC AGC CCC GGA GCT TAC TGT GTT CCT AGT GCA CAG
        R  E  Q  G  S  P  G  A  Y  C  V  P  S  A  Q                 155

507     TGG CCT TGT GCC GCT GGT AGG AAA TAC TAT GGC CGA GGC CCC ATA
        W  P  C  A  A  G  R  K  Y  Y  G  R  G  P  I                 170

552     CAG ATT TCC TAC AAC TAC AAC TAT GGG CAA GCT GGG AAA GCC ATA
        Q  I  S  Y  N  Y  N  Y  G  Q  A  G  K  A  I                 185

597     GGG GTA GAC CTG GTA AAC AAC CCT GAT CTA GTA GCA ACA GAT GCA
        G  V  D  L  V  N  N  P  D  L  V  A  T  D  A                 200

642     GTC ATA TCA TTC AAG ACA GCC TTC TGG TTC TGG ATG ACA CCC CAG
        V  I  S  F  K  T  A  F  W  F  W  M  T  P  Q                 215

687     TCA CCC AAG CCT TCC TGC CAT AAT GTC ATC ACA GGA GGA TGG ACC
        S  P  K  P  S  C  H  N  V  I  T  G  G  W  T                 230

732     CCA TCA GGT GCA GAT AGG TCA GCA GGG CGG CTT CCC GGT TTT GGT
        P  S  G  A  D  R  S  A  G  R  L  P  G  F  G                 245

777     GTT ATC ACA AAC ATC ATC AAT GGA GGT GTT GAA TGT GGG AAA GGG
        V  I  T  N  I  I  N  G  G  V  E  C  G  K  G                 260

822     GTA GTT CCT CAG GTC CAG GAC CGC ATA GGT TTC TAT AAG AGG TAC
        V  V  P  Q  V  Q  D  R  I  G  F  Y  K  R  Y                 275

867     TGT GAT ATA CTT AGG GTT AGC TAT GGC AAT AAC CTG GAC TGC AAC
        C  D  I  L  R  V  S  Y  G  N  N  L  D  C  N                 290

912     AAC CAA AGG CCT TTC GGG TCT GGC CTC CTG CTG GAC ACC ATC TAA
        N  Q  R  P  F  G  S  G  L  L  L  D  T  I  *                 314

957     AGTTGCTGTATGCTTTCTGCTCAACCTGTTTTGTCCACTCTGCTATGGTGATGTGATAA
1015    GTTGTATCCATGAACTGTATGAATAAACGTCTTAGTTGCTACCATCAAACAACTAAGAG
1074    AAGATCAATAATATGCAAAATTTTGTTTTCTGAGCAAAAAAAAAAAAAAAAAA
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Figure 1. cDNA and translated amino acid sequences of *Vitis* basic class I chitinase (VCHITIb). The nucleotide and amino acid residues are numbered in the left and right margins, respectively, and polyadenylation signals are underlined. The first termination codon is marked by an asterisk.

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                ⌈ signal peptide                    ⌉⌈      cysteine-rich domain
Vitis           ..... MGLWALVAFC LLSLIILVGSA --EQCGGQAG GRVCPGGACC SKFGWCGNTA   48
Nicotiana       .....MRL REFT**SSLL FSL*L*SA** --****S*** *AR*AS*L** *********N  51
Solanum          MWCNMRRHK EVNFVAYLLF S*LVLVSAAL -AQN**S*G* *KA*AS*Q-* *********N  57
Populus         ..... *RF***TVLS **LSL*L*VS STA***S*** NAT**NDL** *SG*Y**L*V   50
WGA             ..... ..... ..... ..QR**E*GS *ME**NNL** *QY*Y**MGG            28

                        ⌈ hinge   ⌈ catalytic domain
Vitis           DYCGSG-CQS QCSST----- --GDIGQLIT RSMFNDMLKH RNEGSCPGKG FYTYDAFIAA  100
Nicotiana       ****P*N*** **PGGPTPPG G-**L*SI*S S***DQ**** **DNA*Q*** **S*N***N*  110
Solanum         ******N*** **PGGGPGPG PG**L*SA*S N***DQ**** ***N**Q**N **S*N*IN**  117
Populus         A**CA*-*V* **RNCFF--- ---------* E***EQ**PN **ND****** *-****YFV*   97
WGA             ****K*-**N GACW*SKRCG SQAGGKTCPN NHCCSQYGHC GFGAEYC*A* QGGPCRADIK   87

                                                                              ⌈
Vitis           AKAFPGFGTT GDTTTRKREI AAFLAQTSHE TTGGWASAPD GPYAWGYCYL REQGSPGAYC  160
Nicotiana       *RS*******S ****A**EIA *F*-****** ******T*** ********W* *******D**  169
Solanum         -RS*******S **INA***** ***F****** ********** ********F* **R*N**D**  176
Populus         TEFY****M* **DD*****L ***F****Q* *S*RSIIGE* A*FT****LV N*-LN*NSD*  156
WGA             CGSQA*GKLC PNNLCCSQWG YCG*GSEFCG EGCQNGACST DKPCGKDAGG *VCTNNCCSK  147

          variable region ⌉
Vitis           VPSAQWPCAA GRKYYGRGPI QISYNYNYGQ AGKAIGVDLV RNPDLVATDA VISFKTAFWF  220
Nicotiana       T**G*****P ****F***** ****-_-***P C*R*****L *********P *****S*L**  227
Solanum         P**S-****P ****F***** ***H*****P C*R*****L *********P *******L**  235
Populus         D*KTKSSYPC VAD******L *LRW*****E C*NYL*QN*L DE*EK****P *L**EA*L**  216
WGA             WG*CGIGPGY CGAGCQS*GC DGVFAEAIAT NSTLLAE                          184

Vitis           WMTPQSPKPS CHNVITGGWT PSGADRSAGR LPGFGVITNI INGGVECGKG VVPQVQDRIG  280
Nicotiana       ********** **D**I*R*Q **S***A*N* --********* ****L***R* TDSR******  285
Solanum         ********** **D**I*R*N **S***A*N* ********** ****L***R* TDNR******  295
Populus         **N*HTGA** **E****E*S **E**IE*** K****ML*** *TN*G**T*D GKTRQ*N**D  276

                                                ⌈ C-terminal extension
Vitis           FYKRYCDILR VSYGNNLDCN NQRPFGSGLL LDTI                              314
Nicotiana       **R***S**G **P*D****G ***S**N*** V**M                              319
Solanum         **R***S**G *TP-D****V ***W**NA** V**L                              328
Populus         Y*L****M*Q *-P*D**Y*D **ET*EDNG* *KMVGTM                           312
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Figure 2. Alignment of basic class I chitinase polypeptides from *V. vinifera* (VCHIT1b), *N. tabacum* (Shinshi et al., 1990), *Solanum tuberosum* (Gaynor, 1988), and *Populus tremuloides* (Parsons et al., 1989). The Cys-rich domain was additionally compared with the sequence of wheat germ agglutinin (Rhaikel and Wilkins, 1987). Dashes indicate gaps introduced for maximal alignment, and asterisks replace identical amino acid residues. The catalytic consensus sequence of chitinases (Meins et al., 1992) is printed in bold.

cDNA library with this probe and DNA sequencing selected a clone of 1061 bp, designated VCH3. The insert of this clone contained one long open reading frame of 903 bp encoding a polypeptide of 301 amino acid residues, which was flanked by 19- and 139-bp sequences (Fig. 3). Only one polyadenylation site was detected in the downstream region followed by a short poly(A) tail (Fig. 3), and sequence alignments revealed about 65% identity of the VCH3 cDNA sequence with class IIII chitinase cDNAs from tobacco, cucumber, and Arabidopsis (Metraux et al., 1989; Samac et al., 1990; Stintzi et al., 1990). The relatively low level of homology of VCH3 cDNA with the tobacco sequence explained the failure of cross-hybridization in heterologous screenings. Nevertheless, the antiserum generated to the tobacco class III chitinase recognized the corresponding *V. vinifera* enzyme expressed in *E. coli* (G. Busam, unpublished data) and corroborated the identity of the isolated cDNA.

A molecular mass of 32,349 D was calculated for the translated VCH3 polypeptide (Fig. 3), and also the peptide

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1                                                        ATTCGGCACGAGTGCAAGA

20      ATG GCT AGA ACC CCC CAA TCC ACA CCT CTG CTC ATC TCC CTC TCA
        M  A  R  T  P  Q  S  T  P  L  L  I  S  L  S                  15

65      GTC CTA GCC CTC CTC CAG ACC TCT TAT GCT GGT GGC ATC GCA ATC
        V  L  A  L  L  Q  T  S  Y  A  G  G  I  A  I                  30

110     TAC TGG GGC CAA AAC GGC AAT GAA GGG ACC CTA ACC CAA ACC TGC
        Y  W  G  Q  N  G  N  E  G  T  L  T  Q  T  C                  45

155     AAC ACC GGA AAA TAC TCC TAC GTT AAC ATA GCC TTC CTC AAC AAG
        N  T  G  K  Y  S  Y  V  N  I  A  F  L  N  K                  60

200     TTC GGC AAT GGC CAG ACC CCT GAA ATC AAC CTT GCT GGC CAT TGC
        F  G  N  G  Q  T  P  E  I  N  L  A  G  H  C                  75

245     AAC CCT GCT TCC AAT GGC TGC ACC TCC GTC AGT ACC GGC ATA AGA
        N  P  A  S  N  G  C  T  S  V  S  T  G  I  R                  90

290     AAC TGC CAA AAT CGA GGC ATT AAG GTC ATG CTC TCT ATA GGT GGT
        N  C  Q  N  R  G  I  K  V  M  L  S  I  G  G                 105

335     GGG GCT GGA AGT TAC TCT CTG TCC TCT TCC AAT GAT GCC CAA AAC
        G  A  G  S  Y  S  L  S  S  S  N  D  A  Q  N                 120

380     GTA GCC AAT TAT CTG TGG AAC AAC TTC TTG GGA GGA CAA TCA TCG
        V  A  N  Y  L  W  N  N  F  L  G  G  Q  S  S                 135

425     TCT CGG CCA TTA GGT GAT GCA GTA TTA GAT GGC ATA GAC TTC GAC
        S  R  P  L  G  D  A  V  L  D  G  I  D  F  D                 150

470     ATC GAG CTC GGT TCG ACC CTG CAC TGG GAT GAC CTT GCT CGA GCC
        I  E  L  G  S  T  L  H  W  D  D  L  A  R  A                 165

515     TTA TCC CGT ATC GAG TTT CAG CAA GAG CGT GGA AGG AAG GTA TAC
        L  S  R  I  E  F  Q  Q  E  R  G  R  K  V  Y                 180

560     CTC ACT GCA GCC CCT CAA TGT CCA TTC CCA GAT AAG GTC CCT GGC
        L  T  A  A  P  Q  C  P  F  P  D  K  V  P  G                 195

605     ACT GCC CTT AAC ACA GGC CTT TTT GAC TAT GTC TGG GTG CAA TTC
        T  A  L  N  T  G  L  F  D  Y  V  W  V  Q  F                 210

650     TAT AAC AAT CCC CCA TGC CAG TAT TCT TCT GGC AAC ACC AAT AAC
        Y  N  N  P  P  C  Q  Y  S  S  G  N  T  N  N                 225

695     CTT CTG AAT TCA TGG AAC CGG TGG ACT TCA TCG ATA AAT TCC ACG
        L  L  N  S  W  N  R  W  T  S  S  I  N  S  T                 240

740     GGA TCC TTC ATG GGC CTG CCG GCA TCC TCT GCA GCT GCC GGA AGG
        G  S  F  M  G  L  P  A  S  S  A  A  A  G  R                 255

785     GGG TTT ATT CCG GCC AAT GTG TTG ACC TCT CAA ATT CTT CCG GTC
        G  F  I  P  A  N  V  L  T  S  Q  I  L  P  V                 270

830     ATT AAG AGA TCA CCC AAG TAT GGA GGC GTG ATG CTG TGG TCA AAG
        I  K  R  S  P  K  Y  G  G  V  M  L  W  S  K                 285

875     TAC TAT GAT GAT CAG AGT GGA TAC AGC TCT TCC ATT AAG AGC AGT
        Y  Y  D  D  Q  S  G  Y  S  S  S  I  K  S  S                 300

920     GTG TGA ACTGGGTGTTAGTCTATCTATATCAGTTGTAATAATATATTGAATAAGCTT
        V  *           *                                            301

977     TCATCCTCGATGAATGTATGGATAAAGTGAAAGAGAATATTATGGAGAATATAGTATGA
1036    AGAATTATAAAAAAAAAAAAAAAAAA
```

Figure 3. cDNA and translated amino acid sequences of *Vitis* sp. acidic class III chitinase (VCH3). The nucleotide and amino acid residues are numbered in the left and right margins, respectively, and the single polyadenylation signal is underlined. Two termination codons are marked by asterisks.

```
                                                  ⌈ mature chitinase
Vitis       ---MARTPQS TPLLISLSVL ALLQTSYAGG IAIYWGQNGN EGTLTQTCNT GKYSYVNIAF  57
Hevea       ---------- ---------- ---------* ********** ********S* R*********  32
Cucumis     ----**AHKIT *T*S*FFLLS SIFRS*D*A* ********** **S*AS**A* *N*EF*****  57
Arabidopsis MTN*TLRKHV IYF*FFSCS* SKPSDASR** ********** **N*SA**A* *R*A***V**  60
Nicotiana   ------MIKY SF**TA*VLF LRALKLE**D *V********* **S*AD**A* NN*AI*****  54

Vitis       LNKFGNGQTP EINLAGHCNP ASNGCTSVST GIRNCQNRGI KVMLSIGGGA GSYSLSSSND 117
Hevea       ********** Q********* *AG***I**N ***S**IQ** *-***L***I ***T*A*QA*  91
Cucumis     *-S**S**A* VL******** DN***AFL*D E*NS*KSQNV **L******* *******AD* 116
Arabidopsis *V******** *L******** *AT-**HFGS QVKD**S*** *****L***I *N**IG*RE* 119
Nicotiana   *VV*****N* VL******D* NAGA**GL*N D**A***Q** **-**L**** ***F***AD* 113

Vitis       AQNVANYLWN NFLGGQSSSR PLGDAVLDGI DFDIELGSTL HWDDLARALS RIEFQQERGR 177
Hevea       *K***D**** *****K**** ********** *****H**** Y*******Y-* AYSK*---*K 147
Cucumis     *KQ***FI** SY*****D** ***A*****V *****S**GQ F**V**QE*- -KN*G*---- 170
Arabidopsis *KVI*D**** *****K**** *********- **N*****PQ *******T** ---KFSH*** 175
Nicotiana   *R******** *Y*****NT* ********** *****G*T*Q ***E**KT*Q --FS**R--- 168

Vitis       KVYLTAAPQC PFPDKVPGTA LNTGLFDYVW VQFYNNPPCQ YSSGNTNNLL NSWNRWTSSI 237
Hevea       ********** ****RYL*** ********** ********** *****I**II *******T*- 206
Cucumis     -*I*S***** *I**-AHLA* IK*****S** *********M FAD-*AD*** S***Q**AFP 227
Arabidopsis *I***G**** ****RLM*S* ***KR***** I********S S*G-**Q**F D***R**T** 234
Nicotiana   ********** ****TWLNG* *S******** ********** **G*SAD**K *Y**Q*NAI- 227

Vitis       NSTGSFMGLP ASSAAAGRG- FIPANVLTSQ ILPVIKRSPK YGGVMLWSKY YDDQSGYSSS 295
Hevea       *AGKI*L*** *APE***S*- YV*PD**I*R ***E**K*** *********F ***KN***** 265
Cucumis     T*-KLY**** *ARE**PS*G ****D*I-** V**T**A*SN *********A F*N--***D* 283
Arabidopsis AAQKF*L*** *APE**DS*- Y**PD***** ***TL*K*R* ********FW D*-KN***** 292
Nicotiana   -QGKI*L*** *AQG***S*- ***SD**V** V**L*NG*** *********F **--N****A 283

Vitis       IKSSV                                                            301
Hevea       *LD**                                                            270
Cucumis     **G*IG                                                           289
Arabidopsis *LA**                                                            297
Nicotiana   **AN*                                                            288
```

Figure 4. Alignment of class III chitinase polypeptides from *Vitis* sp. (VCH3), *Hevea brasiliensis* (Jekel et al., 1991), *C. sativus* (Metraux et al., 1989), *Arabidopsis thaliana* (Samac et al., 1990), and *N. tabacum* (Stintzi et al., 1993). Dashes indicate gaps introduced for maximal alignment, and asterisks replace identical amino acid residues. The consensus sequence of the mature chitinase (Meins et al., 1992) is printed in bold.

sequence revealed about 65% sequence identity to class III chitinases from tobacco, cucumber, Arabidopsis, or *H. brasiliensis* (Fig. 4) and complied fully to the consensus sequences proposed for class III chitinases (Meins et al., 1992). VCH3 cDNA encoded a hydrophobic signal peptide of 24 amino acid residues, which is required for the Golgi passage and cellular excretion of the enzyme, and this signal peptide lacked sequence similarity to any of the signal peptides of heterologous class III chitinases or of VCHIT1b (Fig. 4).

Leaf DNA of young *V. vinifera* plants was used for the restriction with the endonucleases *Eco*RI, *Eco*RI/*Pst*I, *Pst*I, or *Hin*dIII, and the Southern hybridization analysis was carried out with digoxigenin-labeled VCHIT1b or VCH3 cDNA probes. The cDNAs hybridized with varying intensities to several bands in each of the restriction digests, suggesting that both the class I and class IIII chitinases of grapevine are encoded by small gene families.

Expression of Chitinases in Response to Elicitation or Chemical Activation of Cells

The systemic expression of chitinases had been observed in some test plants and was regarded as an indicator of the systemic acquired disease resistance response (Ward et al., 1991; Lawton et al., 1992, 1994). Accordingly, the expression of VCHIT1b and VCH3 was studied in grapevine cell cultures in response to treatment with biotic elicitors or low, nontoxic concentrations of SA, INA, or BTH. The elicitation with yeast extract induced the expression of both VCH3 and VCHIT1b transcripts over the 24-h period of the

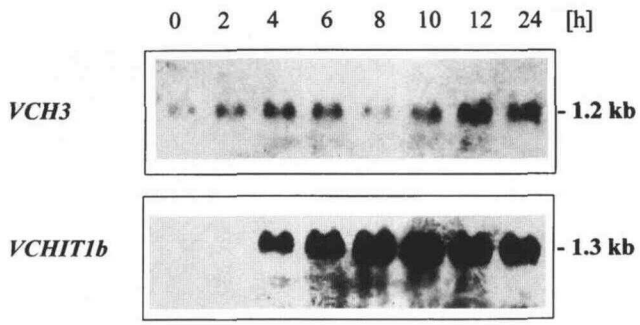

Figure 5. Northern-blot hybridizations of class I (VCHIT1b) and class III (VCH3) chitinase transcripts isolated from *V. vinifera* cells that had been treated for various times with yeast extract (1 mg mL^{-1}). The total RNA of the cells (7.5 μg/lane) was separated, and the blots were developed with digoxigenin-labeled VCHIT1b or VCH3 cDNA probes.

experiments, commencing after a lag of about 1 h (Fig. 5), and the size of the hybridizing bands of about 1.2 and 1.3 kb for VCH3 and VCHIT1b, respectively, suggested an average degree of mRNA polyadenylation. The induction intensities differed considerably, however, showing strong hybridization signals for VCHIT1b and rather weak signals for VCH3. Furthermore, VCHIT1b mRNA appeared to be absent from noninduced cells, whereas significant, albeit low, constitutive expression of VCH3 was observed. In different sets of experiments, an aqueous suspension of live *P. syringae* pv *syringae* cells was used as a second biotic elicitor, which caused the rapid and transient induction of VCH3 mRNA in the grapevine cells with a maximum at 2 h, whereas VCHIT1b mRNA was not significantly induced (Fig. 6). These results convincingly documented that the two grapevine chitinases belong to different regulatory groups of enzymes and can be differentially expressed depending on the quality of the biotic elicitor.

To assess the capabilities of grapevine cells for the SAR response, which is supposedly associated with the differential expression of chitinases as postulated for some other plants (Ward et al., 1991; Lawton et al., 1994), the grapevine cell cultures were treated with INA or BTH. The addition of INA strongly induced the transcription of VCH3 over the 24-h period of the experiments, following a lag of about 1 h, whereas the expression of VCHIT1b remained at low levels (Fig. 6). A very similar pattern of induction was observed in response to the BTH treatment (Fig. 7), and the results resemble those observed in the grapevine cells upon challenge with *P. syringae* pv *syringae*. No induction was observed in control cultures treated with water or with the formulation aid (wettable powder) lacking INA or BTH. As a further control, the grapevine cultures were treated with SA (Fig. 7), and the transiently enhanced amounts of both VCH3 and VCHIT1b transcripts (reaching maximal values at 4 h) were recorded (Fig. 7). The results unequivocally demonstrated that grapevine cells are capable of responding to chemicals such as INA or BTH, which have been proposed as SAR activators (Kessmann et al., 1997), and that the pattern of expression of the class I and class IIII chitinases is directly comparable to the pattern reported for cucumber or tobacco test plants (Ward et al., 1991; Lawton et al., 1994). The differential kinetics of induction moreover suggested that the mode of action of INA and BTH differed from that of SA, and it is unlikely that the SAR activators mimic the action of SA, as had been suggested earlier (Ryals et al., 1996).

Systemic Induction of Chitinases

Single leaves of healthy *V. vinifera* or *V. rupestris* plantlets raised in the greenhouse were inoculated with spore suspensions of *P. viticola*, the causal agent of the downy mildew disease. The inoculated leaves as well as the healthy leaves of the younger, next stage were harvested at various time intervals of incubation for northern probing of chitinase transcripts. The transient, biphasic induction of VCH3 mRNA abundance to maximal values at 2 and 6 d was observed in the inoculated leaf tissue (Fig. 8). *V. vinifera* is susceptible to *P. viticola* infection and macroscopic signs of damage were first visible after 5 d. A corresponding pattern of induction, although of much weaker intensity, was observed in the tissues of the next younger leaf stage (Fig. 8), which were confirmed as being healthy by microscopic examination throughout the experiment (10 d). In contrast to the infected tissue, however, hybridization signals of equivalent intensities were observed at d 6 and 8 for the systemic induction response (Fig. 8). The different kinetics of the systemic versus the local response may be caused by the destruction of the infected leaf tissue, which became obvious after 7 d of incubation. The expression of VCH3 was not induced in control tissues of either leaf stage (Fig. 8, lanes 1 and 8). Very similar observations were made on leaves of *V. vinifera*. plantlets infected with *Uncinula neca-*

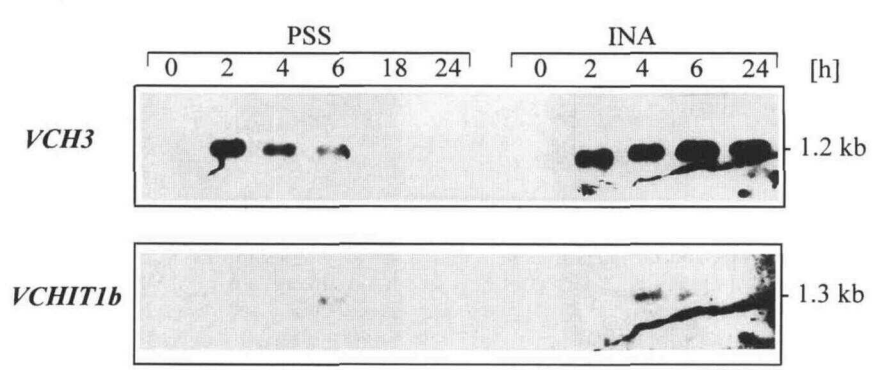

Figure 6. Accumulation of class I (VCHIT1b) and class III (VCH3) chitinase mRNAs following treatment with *P. syringae* pv *syringae* (1×10^9 cells/40 mL culture) or INA (final concentration 25 μM). Poly(A^+) RNA was extracted from *Vitis* cells at various times after the addition of *P. syringae* pv *syringae* or INA and subjected to northern hybridization (0.7 μg/lane) with digoxigenin-labeled VCHIT1b or VCH3 cDNAs probes, respectively.

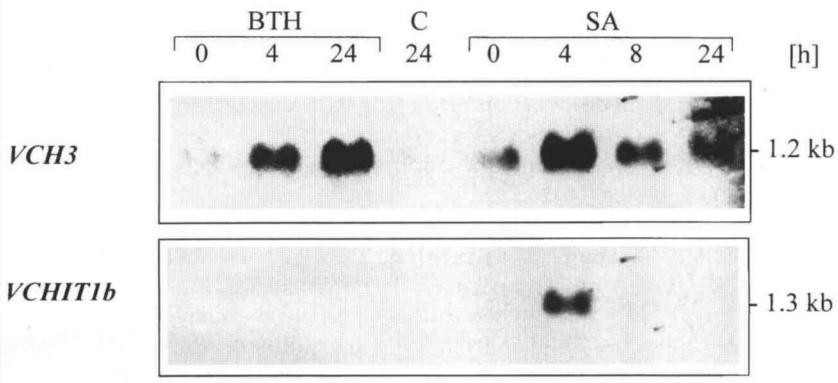

Figure 7. Accumulation of class I (VCHIT1b) and class III (VCH3) mRNAs in *Vitis* sp. cells following treatment with BTH (final concentration 25 μM) or SA (final concentration 20 μM). The total RNA was extracted from the cells at various times after the addition of BTH or SA and subjected to northern hybridization (7.5 μg/lane) with digoxigenin-labeled VCHIT1b and VCH3 cDNA probes. The mock inoculation of cell cultures for 24 h (C24) with the formulation aid lacking an active ingredient (wettable powder) served as a control.

tor, the causal agent of powdery mildew, although much less systemic induction was observed under the experimental conditions (G. Busam, unpublished data). The *P. viticola* inoculation of single leaves of *V. rupestris*, which is resistant to the downy mildew fungus, caused the development of very small necrotic lesions within 4 d and the fungus did not grow any further. The VCH3 transcript abundance increased steeply in the inoculated tissue and culminated with prominent hybridization signals in a transient maximum after 4 d (Fig. 9). The intensity leveled off later, and the induction characteristic resembled those observed with *V. vinifera*. However, no systemic enhancement of VCH3 mRNA was detected over the time of the experiment (7 d; Fig. 9).

In neither *Vitis* sp. did the inoculations cause the significant induction of VCHIT1b mRNA abundance and the constitutive expression of this mRNA stayed rather weak. These data emphasize that the expression of VCH3, but not VCHIT1b, is preferentially modulated in grapevine plants responding to fungal challenge, and grapevine plants are capable of responding systemically to local infections. The differential expression of chitinases may be considered a characteristic factor to evaluate the SAR response in grapevine.

DISCUSSION

The intriguing SAR concept was developed initially for cucumber and tobacco. Although the chemical nature of the systemic signal and the molecular mechanisms involved are still a matter of investigation, the usefulness of the concept for commercial plant protection is being studied, and the synthesis of chemicals such as INA and BTH, which activate the plants' SAR, has spurred the committed research. The systemic induction of lytic enzyme activities was correlated with the expression of SAR (Binder et al., 1989) and the differential expression of chitinases in advance of the fungus was considered a convenient indicator of successful signaling. Grapevine is a rewarding target of SAR research because it is a crop of worldwide economic importance, which is prone to several devastating fungal diseases and requires the frequent application of fungicides (Braun, 1995; Agrios, 1997). The relevant literature, how-

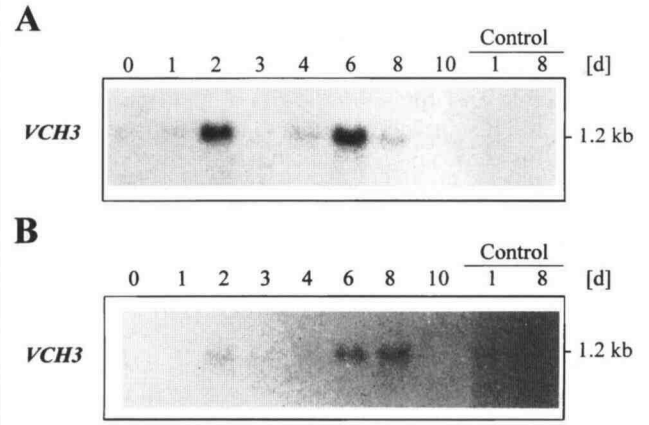

Figure 8. Transcript abundance of class III chitinase (VCH3) in leaf tissues of fungus-infected *V. vinifera* plants. Single lower leaves of the susceptible *V. vinifera* cultivar were inoculated with *P. viticola* (lanes 2, 5×10^4 spores mL^{-1}), and the infected leaves (A) and the younger leaves of the next stage (B) were harvested over the next 10 d for northern blotting of the RNA. Leaves of healthy plants grown in the same growth chamber were harvested at d 1 or 8 and used as a control. The total RNA was extracted and subjected to northern hybridization (10 μg/lane) with the ^{32}P-labeled VCH3 cDNA probe.

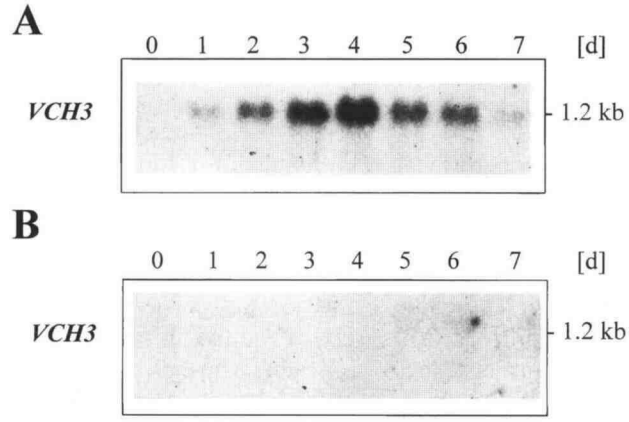

Figure 9. Transcript abundance of class III chitinase (VCH3) in leaf tissues of fungus-inoculated *V. rupestris* plants. Single, lower leaves of the resistant *V. vinifera* sp. cultivar, were inoculated with *P. viticola* (lanes 2, 5×10^4 spores mL^{-1}), and the infected leaves (A) and the younger leaves of the next stage (B) were harvested over the next 7 d for northern blotting of the RNA. The total RNA was extracted and subjected to northern hybridization (10 μg/lane) with the ^{32}P-labeled VCH3 cDNA probe.

ever, has focused mainly on the accumulation of phytoalexins (Langcake and Pryce, 1976; Blaich and Bachmann, 1980; Melchior and Kindl, 1990) rather than on the potential for induced resistance.

In this report, two chitinases with high sequence homologies to heterologous chitinases (Figs. 2 and 4; Meins et al., 1992) were characterized from cell cultures of *V. vinifera* (Figs. 1 and 3). VCHIT1b was grouped to the basic class I chitinases targeted to the vacuole (Neuhaus et al., 1991) and VCH3 was assigned to the class III chitinases that represent bifunctional, lysozymal enzymes (Metraux et al., 1989) and are secreted into the extracellular space (Boller and Metraux, 1988; Jekel et al., 1991). The in situ translation of VCHIT1b and VCH3 in *Vitis* sp. cells was verified by western-blot cross-reactions with rabbit antisera raised to tobacco class I and class III chitinases and the immunodetection revealed masses of about 29 and 28 kD for the mature class I and III grapevine chitinases (G. Busam, unpublished). Furthermore, in comparison with the polypeptides translated from the cDNAs (Figs. 1–4), the data demonstrated that both chitinases had been processed posttranslationally. The DNA restriction patterns observed on Southern blotting suggested that the grapevine chitinases are encoded by small gene families, which is reminiscent of the class III chitinases in cucumber (Lawton et al., 1994). These findings are supported by the very recent IEF and PAGE distinction of up to 13 forms of chitinases from various grapevine tissues (Derckel et al., 1996), some of which were induced after SA treatment.

Low, albeit significant, levels of VCH3 transcripts were expressed constitutively in the grapevine culture (Fig. 5), in contrast to the tissues of differentiated plants (Figs. 8 and 9). This might be attributed to the hormone status, since chitinase expression in tobacco is known to be regulated by auxin or cytokinins (Shinshi et al., 1987). Treatment of *V. vinifera* cell cultures with yeast elicitor considerably enhanced the abundance of both chitinase mRNAs, although the effect was less pronounced on VCH3 (Fig. 5) and the observed enhancement suggested the induction of de novo transcription. In contrast, treatment of the cells with INA or BTH, which were characterized as SAR activators (Kessmann et al., 1997), increased exclusively the amounts of the VCH3 transcripts (Figs. 6 and 7) and basically the same effects were observed upon treatment of the cell cultures with live *P. syringae pv syringae* (Fig. 6). INA and BTH had been considered to mimic the action of SA (Ryals et al., 1996). Control experiments, however, clearly revealed the diverse responses of the *Vitis* sp. cells upon SA or INA and BTH treatments (Figs. 6 and 7). Overall, the results suggested that *Vitis* sp. basic class I chitinase is not associated with the response to SAR activators, which is in accordance with reports for Arabidopsis or tobacco (Ward et al., 1991; Uknes et al., 1992). The class III chitinase, on the other hand, was evidently up-regulated in response to bacterial or fungal elicitation (Figs. 5 and 6) as well as to the chemical activation by INA and BTH (Figs. 6 and 7), and the expression of VCH3 appeared synonymous with the activation of disease resistance in grapevine. These data are fully compatible with the results reported previously for cucumber (Lawton et al., 1994) and corroborated the idea that grapevine cells are capable of responding to plant activators (Kessmann et al., 1997) analogously to cucumber, tobacco, and Arabidopsis test plants. Moreover, the mode of action of INA or BTH differed substantially from the action of SA as documented by the divergent induction kinetics and the additional rapid induction of VCHIT1b (Figs. 6 and 7).

In conclusion, the results suggested that INA and BTH act more selectively than SA. Plants like tobacco metabolize SA rapidly, particularly by glucosidic conjugation (Lee et al., 1995), whereas the conjugate *N*-salicyloylaspartic acid was isolated from *Vitis* sp. cultivars (Rapp and Ziegler, 1973; Steffan et al., 1988). Furthermore, inoculation of *V. vinifera* with *P. viticola* reportedly induced gentisic acid 5-*O*-β-glucoside rather than salicylic conjugates (Weber, 1992), and the physiological relevance of SA and gentisic acid for the SAR response of grapevine needs further research.

Healthy, young plants of *V. vinifera* L. cv Pinot Noir, a cultivar that is highly susceptible to powdery or downy mildew infections, and of the resistant species *V. rupestris* were used to assess the systemic induction of chitinase expression through different leaf stages. Low levels of VCHIT1b transcripts were constitutively expressed in these plants, whereas VCH3 transcripts were virtually absent from unchallenged leaves (Figs. 8 and 9). The inoculation of single, lower-stage leaves with *P. viticola* did not cause significant changes of the VCHIT1b expression in either the inoculated or the next stage, healthy leaves. However, prominent changes in the abundance of VCH3 transcripts were induced in the inoculated leaves of *V. vinifera.*, resulting in two transient maxima at 2 and 6 d (Fig. 8). The reason for the biphasic induction is unclear and might reflect different growth or sporulation stages of the fungus. More importantly, however, the abundance of VCH3 mRNA increased also transiently in the healthy tissue of the younger, next-stage leaf (Fig. 8), which strongly suggested that grapevine is capable of generating the SAR response. The interaction of *P. viticola* with *V. rupestris* is incompatible and the containment of the fungus in tiny necrotic lesions in the inoculated leaves (hypersensitive response) was very effective. Coincidentally with the development of necrotic lesions, a transient maximum of VCH3 mRNA was observed (Fig. 9), whereas no VCH3 transcript could be detected in the healthy tissue of the next-leaf stage (Fig. 9). However, the systemic induction of resistance is not necessarily linked to hypersensitivity (Delaney, 1997) and might appear unnecessary under the short-term exposure of plant tissues in this specific pathogen interaction. The conclusions drawn from the inoculation studies are in accord with the pertinent literature (Ward et al., 1991; Lawton et al., 1994; Ryals et al., 1996) and support the concept of systemic differential PRP induction likely concomitantly with the SAR response for grapevine plants. The concept was further supported by preliminary observations of *V. vinifera* or *V. rupestris* plants sprayed with the SAR activator BTH, which again predominantly induced the VCH3 mRNA (G. Busam, unpublished data).

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The GenBank/EMBL accession numbers for the sequences of *Vitis* basic class I (VCHIT1b) and class III (VCH3) chitinases are Z54234 and Z68123, respectively.

LITERATURE CITED

Agrios GN (1997) Plant Pathology, Ed 4. Academic Press, San Diego, CA

Bernasconi P, Locher R, Pile PE, Jolles P (1987) Purification and N-terminal amino-acid sequence of a basic lysozyme from *Parthenocissus quinquifolia* cultured *in vitro*. Biochim Biophys Acta **915:** 254–260

Binder A, Baer G, Hofmann C, Kovats K (1989) Mechanisms in systemic induced disease resistance. NATO ASI Adv Sci Inst Ser Ser H Cell **36:** 267–272

Blaich R, Bachmann O (1980) The resveratrol synthesis in *Vitaceae*. Induction and cytological observations. Vitis **19:** 230–240

Boller T, Metraux JP (1988) Extracellular localization of chitinase in cucumber. Physiol Mol Plant Pathol **33:** 145–147

Braun U (1995) The Powdery Mildew (*Erysiphales*) of Europe. Gustav Fischer Verlag, Jena, Germany

Busam G, Junghanns KT, Kneusel RE, Kassemeyer H-H, Matern U (1997) Characterization and expression of caffeoyl-CoA 3-*O*-methyltransferase proposed for the induced resistance response of *Vitis vinifera* L. Plant Physiol **115:** 1039-1048

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep **11:** 113–116

Delaney TP (1997) Genetic dissection of acquired resistance to disease. Plant Physiol **113:** 5–12

Derckel JP, Legendre L, Audran JC, Haye B, Lambert B (1996) Chitinases of the grapevine (*Vitis vinifera* L.): five isoforms induced in leaves by salicylic acid are constitutively expressed in other tissues. Plant Sci **119:** 31–37

Friedrich L, Lawton K, Ruess W, Masner P, Specker N, Gut Rella M, Meier B, Dincher S, Staub T, Uknes S, and others (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. Plant J **10:** 61–70

Fukuda Y, Shinshi H (1994) Characterization of a novel *cis*-acting element that is responsive to a fungal elicitor in the promoter of a tobacco class I chitinase gene. Plant Mol Biol **24:** 485–493

Gaffney T, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261:** 754–756

Gaynor JJ (1988) Primary structure of an endochitinase mRNA from *Solanum tuberosum*. Nucleic Acids Res **16:** 5210

Goldsbrough AP, Albrecht H, Stratford R (1993) Salicylic acid-inducible binding of a tobacco nuclear protein to a 10 bp sequence which is highly conserved amongst stress-inducible genes. Plant J **3:** 563–571

Graham LS, Sticklen MB (1994) Plant chitinases. Can J Bot **72:** 1057–1083

Hankanes B, Kvam C (1993) Application of magnetic beads in bioassays. Biotechnology **11:** 60–63

Jekel PA, Hartmann BH, Beintema JJ (1991) The primary structure of hevamine, an enzyme with lysozyme/chitinase activity from *Hevea brasiliensis* latex. Eur J Biochem **200:** 123–130

Joosten M, de Wit P (1989) Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as beta-1,3-glucanases and chitinases. Plant Physiol **89:** 945–951

Kauffmann S, Legrand M, Geoffry P, Fritig B (1987) Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins have 1,3-β-glucanase activity. EMBO J **6:** 3209–3212

Kessmann H, Oostendorp M, Ruess W, Staub T, Kunz W, Ryals J (1996) Systemic activated resistance—a new technology for plant disease control. Pestic Outlook **7:** 10–13

Kessmann H, Staub T, Hofmann C, Maetzke T, Herzog J, Ward E, Uknes S, Ryals J (1994) Induction of systemic acquired disease resistance in plants by chemicals. Annu Rev Phytopathol **32:** 439–459

Kombrink E, Schröder M, Hahlbrock K (1988) Several "pathogenesis-related" proteins in potato are 1,3-β-glucanases and chitinases. Proc Natl Acad Sci USA **85:** 782–786

Kombrink E, Somssich IE (1995) Defense responses of plants to pathogens. Adv Bot Res **21:** 1–34

Kuc J (1982) Induced immunity to plant disease. Bioscience **32:** 854–860

Langcake P, Pryce RJ (1976) The production of resveratrol by *Vitis vinifera* and other members of the *Vitaceae* as a response to infection or injury. Physiol Plant Pathol **9:** 77–85

Lawton K, Ward E, Payne G, Moyer M, Ryals J (1992) Acidic and basic class III chitinase mRNA accumulation in response to TMV infection of tobacco. Plant Mol Biol **19:** 735–743

Lawton KA, Beck J, Potter S, Ward E, Ryals J (1994) Regulation of cucumber class III chitinase gene expression. Mol Plant Microbe Interact **7:** 48–57

Lawton KA, Friedrich L, Hunt M, Weymann K, Kessmann H, Staub T, Ryals J (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. Plant J **10:** 71–82

Lee H, Leon J, Raskin I (1995) Biosynthesis and metabolism of salicylic acid. Proc Natl Acad Sci USA **92:** 4076–4079

Legrand M, Kauffmann S, Geoffroy P, Fritig B (1987) Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci USA **84:** 6750–6754

Linthorst HJM (1991) Pathogenesis-related proteins of plants. Crit Rev Plant Sci **10:** 123–150

Majeau N, Trudel J, Asselin A (1990) Diversity of cucumber chitinase isoforms and characterization of one seed basic chitinase with lysozyme activity. Plant Sci **68:** 9–16

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science **250:** 1002–1004

Malamy J, Klessig DF (1992) Salicylic acid and plant disease resistance. Plant J **2:** 643–654

Mauch F, Mauch-Mani B, Boller T (1988) Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol **88:** 936–942

Meins F, Neuhaus JM, Sperisen C, Ryals J (1992) The primary structure of plant pathogenesis-related glucanohydrolases and their genes. *In* T Boller, F Meins, eds, Genes Involved in Plant Defense. Springer Verlag, New York, pp 245–282

Melchers LS, Apotheker-de Groot M, van der Knaap JA, Ponstein AS, Sela-Buurlage MB, Bol JF, Cornelissen BJC, van den Elzen PJM, Linthorst HJM (1994) A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity. Plant J **5:** 469–480

Melchior F, Kindl H (1990) Grapevine stilbene synthase cDNA only slightly differing from chalcone synthase cDNA is expressed in *Escherichia coli* into a catalytically active enzyme. FEBS Lett **268:** 17–20

Metraux JP, Ahl-Goy P, Staub T, Speich J, Steinemann A, Ryals J, Ward E (1991) Induced systemic resistance in cucumber in response to 2,6-dochloroisonicotinic acid and pathogens. *In* H Hennecke, DPS Verma, eds, Advances in Molecular Genetics of Plant-Microbe Interactions. Kluwer Academic Press, Dordrecht, The Netherlands, pp 432–439

Metraux JP, Burkhardt W, Moyer M, Dincher S, Middlesteadt W, Williams S, Payne G, Carnes M, Ryals J (1989) Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. Proc Natl Acad Sci USA **86:** 896–900

Neuhaus JM, Sticher L, Meins F, Boller T (1991) A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. Proc Natl Acad Sci USA **88:** 10362–10366

Ohashi Y, Ohshima M (1992) Stress-induced expression of genes for pathogenesis-related proteins in plants. Plant Cell Physiol **33:** 819–826

Parsons TJ, Bradshaw HD, Gordon MP (1989) Systemic accumulation of specific mRNAs in response to wounding in poplar trees. Proc Natl Acad Sci USA **86:** 7895–7899

Pierpoint WS, Jackson PJ, Evans RM (1990) The presence of thaumatin-like protein, a chitinase and a glucanase among the pathogenesis-related proteins of potato (*Solanum tuberosum*). Physiol Mol Plant Pathol **36:** 325–338

Raikhel NV, Wilkins TA (1987) Isolation and characterization of a cDNA clone encoding wheat germ agglutinin. Proc Natl Acad Sci USA **84:** 6745–6749

Rapp A, Ziegler A (1973) Analysis of the phenolcarboxylic acids (hydroxybenzoic acids and hydroxycinnamic acids) in grape leaves, berries, and wine by micro-polyamide thin-layer chromatography. Vitis **12:** 226–236

Ryals J, Neuenschwander U, Willits M, Molina A, Steiner H-Y, Hunt M (1996) Systemic acquired resistance. Plant Cell **8:** 1809–1819

Samac D, Hironaka CM, Yallaly PE, Shah DM (1990) Isolation and characterization of the genes encoding basic and acidic chitinase in *Arabidopsis thaliana*. Plant Physiol **93:** 907–914

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-termination inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Schlumbaum A, Mauch F, Vögeli U, Boller T (1986) Plant chitinases are potent inhibitors of fungal growth. Nature **324:** 365–367

Sela-Buurlage MB, Ponstein AS, Vloemans SA, Melchers LS, Van den Elzen PJM, Cornelissen BJC (1993) Only specific chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiol **101:** 857–863

Shinshi H, Mohnen D, Meins F (1987) Regulation of a plant pathogenesis-related enzyme: inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc Natl Acad Sci USA **84:** 89–93

Shinshi H, Neuhaus JM, Ryals J, Meins F (1990) Structure of a tobacco endochitinase gene: evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine-rich domain. Plant Mol Biol **14:** 357–368

Steenkamp J, Wiid I, Lourens A, van Helden P (1994) Improved method for DNA extraction from *Vitis vinifera* L. Am J Enol Vitic **45:** 102–106

Steffan H, Ziegler A, Rapp A (1988) N-Salicyloyl-aspartic acid: a new phenolic compound in grapevines. Vitis **27:** 79–86

Stintzi A, Geoffroy P, Bersuder D, Fritig B, Legrand M (1993) cDNA cloning and expression studies of tobacco class III chitinase-lysozymes. *In* B Fritig, M Legrand, eds, Mechanisms of Plant Defense Responses. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 312–315

Uknes S, Mauch-Mani B, Ward E, Ryals J (1992) Acquired resistance in *Arabidopsis*. Plant Cell **4:** 645–656

Van Loon LC (1985) Pathogenesis-related proteins. Plant Mol Biol **4:** 111–116

Ward ER, Uknes S, Williams SC, Dincher SS, Metraux JP, Ryals J (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3:** 1085–1094

Weber B (1992) Phenolische Komponenten des Weinrebenblattes: Identität und phythopathologische Bedeutung. PhD thesis. Universität Zürich, Switzerland

Zhou MY, Yue D, Gomez-Sanchez P, Gomez-Sanchez CE (1994) Improved downward capillary transfer for blotting of DNA and RNA. Biotechniques **16:** 58–59